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Efficient delivery of Cre-recombinase to neurons in vivo and stable transduction of neurons using adeno-associated and lentiviral vectors

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Abstract

Background: Inactivating genes in vivo is an important technique for establishing their function in the adult nervous system. Unfortunately, conventional knockout mice may suffer from several limitations including embryonic or perinatal lethality and the compensatory regulation of other genes. One approach to producing conditional activation or inactivation of genes involves the use of Cre recombinase to remove loxP-flanked segments of DNA. We have studied the effects of delivering Cre to the hippocampus and neocortex of adult mice by injecting replication-deficient adeno-associated virus (AAV) and lentiviral (LV) vectors into discrete regions of the forebrain.

Results: Recombinant AAV-Cre, AAV-GFP (green fluorescent protein) and LV-Cre-EGFP (enhanced GFP) were made with the transgene controlled by the cytomegalovirus promoter. Infecting 293T cells in vitro with AAV-Cre and LV-Cre-EGFP resulted in transduction of most cells as shown by GFP fluorescence and Cre immunoreactivity. Injections of submicrolitre quantities of LV-Cre-EGFP and mixtures of AAV-Cre with AAV-GFP into the neocortex and hippocampus of adult Rosa26 reporter mice resulted in strong Cre and GFP expression in the dentate gyrus and moderate to strong labelling in specific regions of the hippocampus and in the neocortex, mainly in neurons. The pattern of expression of Cre and GFP obtained with AAV and LV vectors was very similar. X-gal staining showed that Cre-mediated recombination had occurred in neurons in the same regions of the brain, starting at 3 days post-injection. No obvious toxic effects of Cre expression were detected even after four weeks post-injection.

Conclusion: AAV and LV vectors are capable of delivering Cre to neurons in discrete regions of the adult mouse brain and producing recombination.

Background

Mice carrying conventional null mutations of genes have been very useful in studying the roles of various molecules in the development and functions of the nervous system. However, the possibility of compensatory changes during development is often raised as an explanation for the very limited phenotypic abnormalities observed in some knockout mice. Furthermore conventional null mutations of genes, which have important functions during development often show an embryonic lethal phenotype, making experiments on adult animals impossible. The ability to manipulate the genotype *in vivo* provides major opportunities for studying gene function in the mammalian nervous system and for developing novel therapeutic strategies [1,2]. A major goal for gene therapies for neurological diseases and injuries is the development of methods for the delivery of transgenes without toxic side effects. Viruses allow efficient gene delivery, even to postmitotic cells, but have inherent toxic effects. Several DNA viruses have been modified in a variety of ways to produce vectors for gene transfer with reduced toxicity [3]. In recent years, two vectors in particular, recombinant adeno-associated virus (AAV) and lentivirus (LV) have been shown to have the potential to mediate the delivery and stable transduction of genes to both dividing and non-dividing cells in the nervous system, without inducing immune responses [4-10]. These characteristics have made such vectors particularly useful for transferring a gene of interest into the CNS.

Over the last few years, development of the Cre-loxP system, which is concerned with recombination mechanisms in the bacteriophage P1, has provided powerful technologies for the manipulation of genes. Cre is a 38 kDa recombinase and loxP is a Cre-specific recognition sequence present in bacteriophage DNA, to which the recombinase binds. This results in the excision or inversion of stretches of DNA flanked by loxP sites, depending on the orientation of the loxP sequences [11]. This process does not require any additional co-factors or accessory proteins [12]. Engineering mammalian genes with loxP signal sequences bracketing key exons or regulatory regions thus offers opportunities for activating, inactivating or replacing specific genes of interest [13-16]. However, Cre has been reported to have toxic effects on cells *in vitro* and *in vivo* causing a reduction in their proliferation and inducing chromosomal aberrations [17-19]. Virally-delivered Cre has been reported to cause cavity formation in adult mouse brain [17]. A possible explanation for the toxicity is the presence of pseudo-loxP sequences in the genomes of mammals, yeast and *E. coli*, which function as a target for Cre [20-23].

Several strains of mice exist which have been engineered to express Cre under the control of promoters with various tissue specificities. These can be bred with animals

carrying loxP-flanked genes to produce gene inactivation in different tissues. However, there are clear advantages to being able to deliver Cre to specific groups of neurons in adult animals. We describe here the construction of replication deficient adeno-associated virus (AAV) and lentivirus (LV) vectors expressing Cre (AAV-Cre and LV-Cre-EGFP) and the efficiency of transduction, effects on cell survival and efficiency of recombination in Rosa26 reporter mice, 3 days and 1, 2 and 4 weeks following stereotactic injections of the vectors into the neocortex and hippocampus. Rosa26 mice carry a copy of the lacZ (β -galactosidase) gene as a reporter for Cre activity, controlled by the Rosa26 locus. Expression of the gene is activated by Cre-driven removal of a stop-cassette, which consists of the neo-expression cassette followed by three pA sequences, flanked by loxP sites [24]. We have compared LV and AAV vectors because AAV vectors can only accept short inserts (approximately 5 kb), which rules out its using some cell type-specific promoters. Our studies demonstrate that viral delivery of Cre can be performed precisely and recombination can be achieved efficiently in the mammalian nervous system without obvious toxic effects.

Results

Virally delivered Cre to a cell line

The primary objective of this part of the study was to determine whether the LV-Cre-EGFP and AAV-Cre viruses allow the delivery of Cre recombinase to mammalian cells. 293T cells were infected either with LV-Cre-EGFP or AAV-Cre viruses. Cells infected with LV-Cre-EGFP were analysed for GFP fluorescence and immunohistochemistry for Cre with Cy3 conjugated secondary antibodies, 16-48 hours after infection. Green fluorescence was detected in the nucleus of many cells and colocalized with Cre (Fig. 1A and 1B). Cultures infected with AAV-Cre also showed many Cre-positive cells, with the enzyme predominantly localized in the nucleus (Fig. 1C). Uninfected cells showed no immunoreactivity for Cre (Fig. 1D).

Virally-delivered GFP is expressed in the adult mouse brain

To verify the efficiency of gene delivery to the brain using our viral vectors, GFP immunohistochemistry was performed on coronal sections of Rosa26 mouse brain 4 weeks after injection of either a mixture of AAV-Cre and AAV-GFP or of LV-Cre-EGFP viruses (Fig. 2). GFP was most strongly expressed in granule cells of the dentate gyrus, and was also strongly expressed in CA2, the terminal part of the CA3 region and in scattered cells in the CA1 region of the hippocampus. Moderate to strong labelling was found in the polymorphic layer of the dentate gyrus (the hilus) (arrows in Fig. 2B and 2D), molecular layer of the dentate gyrus (the suprapyramidal blade) (arrowheads in Fig. 2B and 2D) and the stratum lacunosum-moleculare (arrowheads in Fig. 2A and 2C). GFP-positive

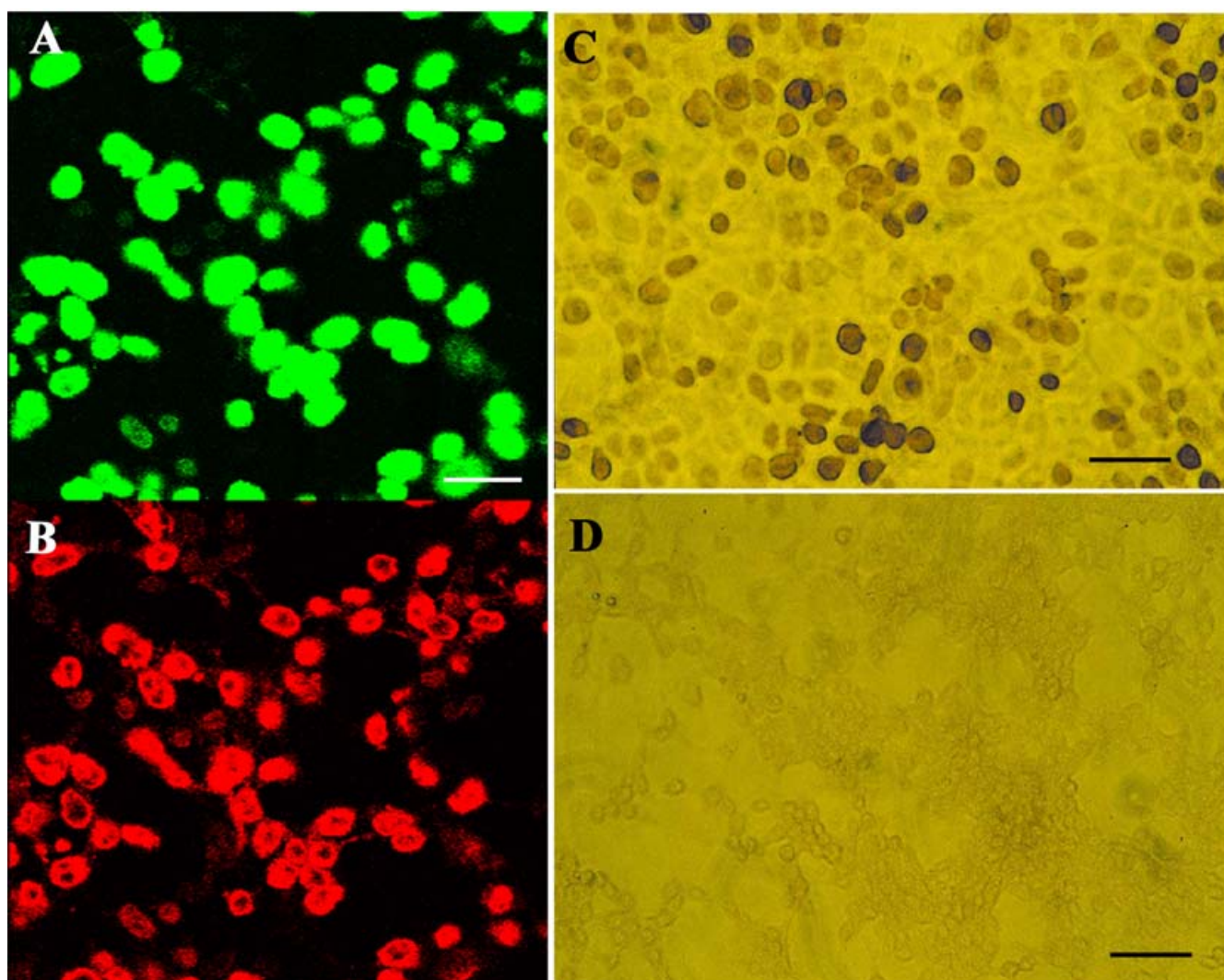


Figure 1

(A, B) EGFP fluorescence (A) and Cre immunoreactivity (B) in HEK 293T cells infected with LV-Cre-EGFP recombinant virus in vitro. All the cells which express EGFP also express Cre. (C, D) Immunohistochemistry for Cre, in HEK 293T cells 16 hrs after exposure to the AAV-Cre virus (1C) and in uninfected HEK 293T cells (1D). Cre is present in the nucleus of large numbers of cells infected with the virus but is absent from uninfected cells. Scale bars A and B = 25 μ m, C = 50 μ m, D = 100 μ m.

cells were also observed in subcortical white matter and corpus callosum (arrows in Fig. 2A and 2C). Strong labelling of the GFP-positive fibres in the molecular layer of the dentate gyrus was also observed (Fig. 2A,2B,2C,2D).

Most cells in mouse brain which express virally-delivered GFP also express Cre

In order to make sure that GFP expression was a good marker for Cre expression in infected cells following LV-Cre-EGFP and AAV-Cre/AAV-GFP mixture injection into Rosa26 mice, immunofluorescence images of sections immunoreacted for both GFP and Cre were compared

(Fig. 3). Following injection of LV or AAV vectors, the patterns of Cre and GFP expression were very similar (compare Fig. 3A with 3B and Fig. 3C with 3D). More cells were Cre positive than EGFP positive, presumably because the detection protocol for Cre was more sensitive. Following injection of AAV-Cre/AAV-GFP mixture, most cells that were immunoreactive for GFP were also Cre positive suggesting that expression of the marker gene and Cre are controlled by similar influences. However, the levels of GFP and Cre varied, so that a few cells had strong GFP and little or no Cre and a few others had strong Cre with little or no GFP (Fig. 3E,3F, and 3G), presumably because not

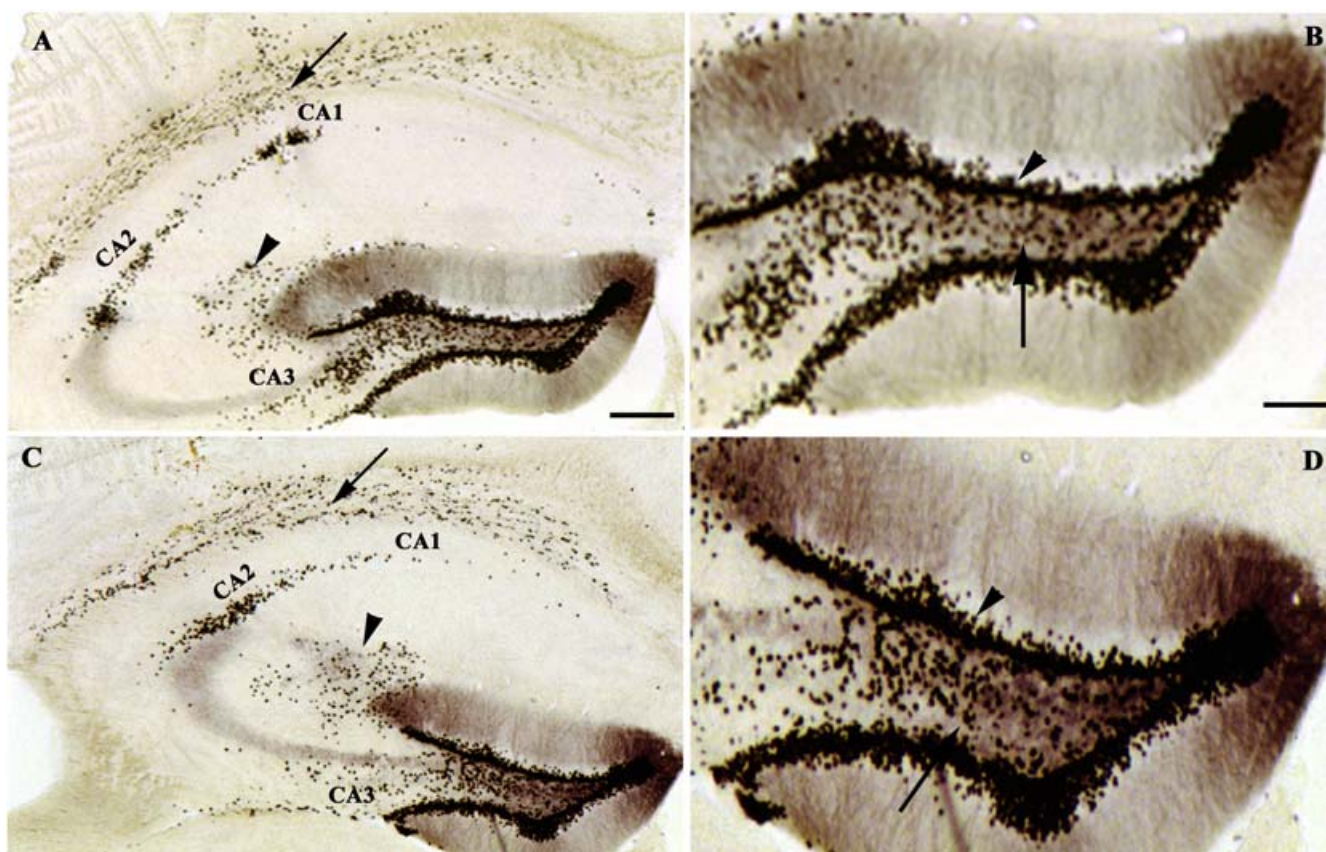


Figure 2

Immunohistochemistry for GFP after infusing LV-Cre-EGFP virus (A, B) and AAV-Cre/AAV-GFP mixture viruses (C, D) into the neocortex and hippocampus of Rosa26 mouse brain. Very strong GFP expression is present in the molecular layer and polymorphic layer (arrows in B and D) of the dentate gyrus. GFP positive cells are present in CA1, CA2 and terminal part of the CA3 region of the hippocampus (A, C). Strong staining was also observed in presumptive glial cells in the subcortical white matter and corpus callosum (arrows in A and C). Fig. 2B and 2D are enlargements of Fig. 2A and 2C. Scale bars, A and C = 200 μ m, B and D = 100 μ m.

all cells took up similar quantities of both vectors. Cre delivered by LV-Cre-EGFP and AAV-Cre was identified in the nucleus of neurons.

Absence of toxic effects of gene delivery with viral vectors

No signs of cavity formation or other obvious tissue damage were found in regions where GFP or Cre were detected following injection of either AAV-Cre/AAV-GFP mixture, or LV-Cre-EGFP viruses into mouse brain. Individual neocortical neurons infected with AAV-Cre/AAV-GFP mixture and LV-Cre viruses could be identified and they retained their normal morphology up to 4 weeks after injection of the vectors.

Virally-delivered Cre produces recombination in adult mouse brain

To show that the Cre recombinase delivered to mouse brain was functional, the pattern of Cre-mediated recombination was studied in adult Rosa26 reporter mice, injected with either LV-Cre-EGFP or AAV-Cre/AAV-GFP mixture. Rosa26 cells express β -galactosidase following Cre-induced recombination. We performed β -galactosidase enzyme histochemistry, 3 days and 1, 2 and 4 weeks following the stereotactic injection of LV-Cre-EGFP and AAV-Cre/AAV-GFP mixture into the neocortex and hippocampus. These two regions were selected to establish the potential of viruses to deliver functionally active Cre to different cell types. β -galactosidase activity was detected in dentate gyrus at all studied time points, but was maximal 2 and 4 weeks following viral injections into the cortex and hippocampus (Figs. 4 and 5). Very strong signal

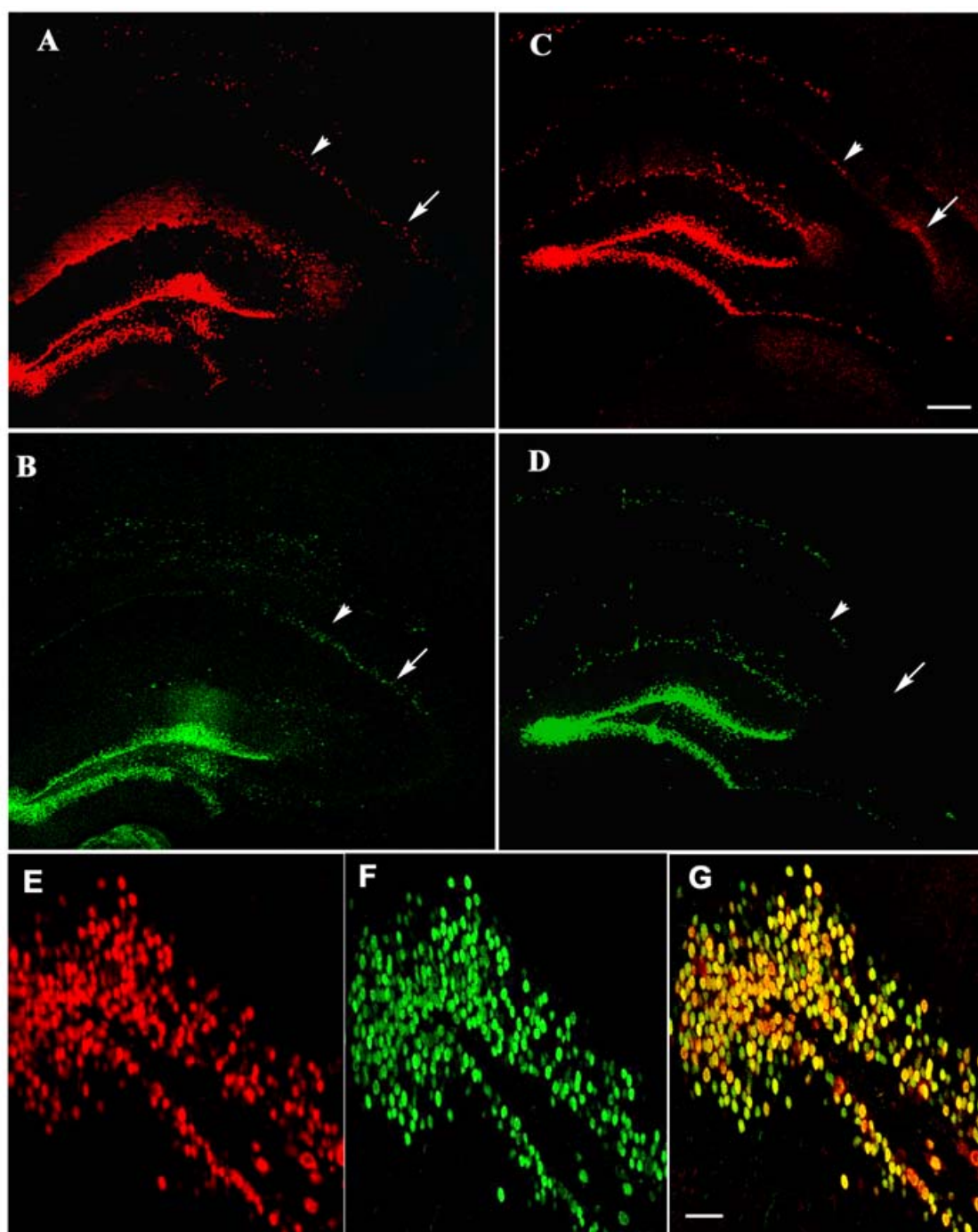


Figure 3

Double immunofluorescence for Cre and GFP using Cy3 and FITC labelled secondary antibodies respectively with single excitations of 543 nm to detect Cre (A, C) or 488 nm to detect GFP (B, D). LV-Cre-EGFP virus (A-B) and AAV-Cre/AAV-GFP mixture viruses (C-D) were injected into the hippocampus and neocortex of Rosa26 mice 4 weeks before perfusion. The patterns of expression of the two transgenes are almost identical (allowing for the slightly stronger staining obtained with the tyramide-enhanced Cre immunohistochemistry) irrespective of the vector used. Fig. 3E,3F, and 3G show the extent of colocalization of GFP and Cre in the dentate gyrus of an AAV-Cre/AAV-GFP mixture injected Rosa26 mouse brain. In the original micrographs, more cells were Cre positive: 373 cells were Cre positive and 256 cells were GFP positive. However the few green cells in 3G show that some cells expressed GFP alone. The arrowheads and arrows in A-D indicate immunofluorescence in pyramidal cells of CA1 and CA2 respectively. Scale bars, A-D = 200 μm, E-G = 400 μm.

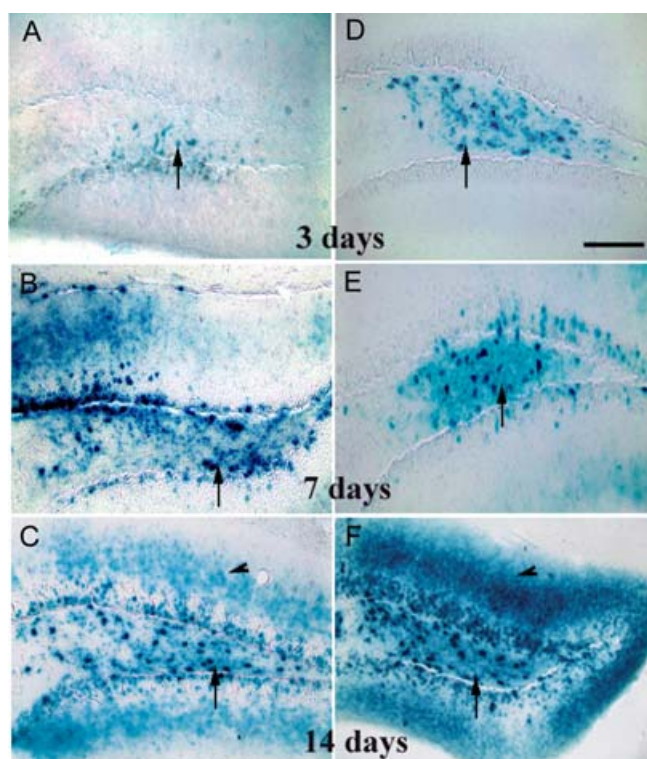


Figure 4
Cre-mediated recombination following injection of LV-Cre-EGFP (A-C) and a mixture of AAV-Cre and AAV-GFP recombinant viruses (AAV-Cre/AAV-GFP mixture) (D-F) into the neocortex and hippocampus of Rosa26 reporter mice. Expression of β -galactosidase, indicating Cre-mediated excision of the loxP flanked stop signal, was detected by the X-gal staining. β -galactosidase activity was prominent in the dentate gyrus of animals at all postinjection intervals (arrows in A-F). Scale bars A= 100 μ m (applies also to B-F).

was found in the granule cell layer of the dentate gyrus, CA2, the terminal portion of CA3 and in the CA1 region of the hippocampus (Fig. 5A and 5D). Moderate to strong labelling was observed in the stratum lacunosum-moleculare (arrowheads in Fig. 5A and 5D), polymorphic layer of the dentate gyrus (the hilus) (arrows in Fig. 4D, 4E, 4F and in Fig. 5C and 5F), and molecular layer of the dentate gyrus (the suprapyramidal blade) (arrowheads in Fig. 4C and 4F and Fig. 5C and 5F). Cre-mediated recombination was first detected in the dentate gyrus and gradually spread to the terminal portion of CA3 (never the whole extent of CA3), CA2 and CA1 regions of the hippocampus. The pattern of recombination, assessed by β -galactosidase activity, in LV-Cre-EGFP injected mice was similar to that produced by injection of AAV-Cre/AAV-GFP mixture. Up to 3 weeks post injection, there were usually more cells showing recombination following injection of AAV-Cre/

AAV-GFP mixture. To determine changes in the number of neurons showing recombination, the number of β -galactosidase-labelled cells in the hilar region of the dentate gyrus was counted in 4–5 sections of brain at each time point examined. At 3 days post injection 47 ± 9.3 (S.D.) were labelled using AAV and 5 ± 8.9 using LV; at 7 days post injection 68 ± 13.5 cells were labelled using AAV and 40 ± 5 using LV; at 2 weeks 83 ± 11.3 cells were labelled using AAV and 50 ± 8.2 using LV; and at 4 weeks 214 ± 10.7 cells were labelled using AAV and 191 ± 28.8 using LV. β -galactosidase was also observed in and around the needle track after viral delivery of Cre (Fig. 5 arrows in A and D). There were some scattered cells in the molecular layer of the dentate gyrus (the suprapyramidal blade) and the stratum lacunosum-moleculare. These were probably interneurons, but one cannot exclude the possibility of non-neuronal cells as well (arrowheads in Fig. 5B and 5E).

Injections of LV-Cre-EGFP and AAV-Cre/AAV-GFP mixture viruses into neocortex and hippocampus also resulted in recombination in glial cells, most obviously in the white matter of the subcortical white matter and corpus callosum (arrows in Fig. 5B and 5E) but the numbers of labelled cells were much lower than the number of nonneuronal cells in the same region which were GFP-positive (compare Figs. 2 and 5).

Discussion

We have successfully used AAV and LV to deliver Cre recombinase to a mammalian cell line in vitro and to cells in the brains of Rosa26 mice 3 days to 4 weeks after stereotactic injection in the neocortex and hippocampus. The AAV and LV vectors produced stable transduction of large numbers of neural cells in the regions injected and produced recombination in those cells without obvious tissue damage. These vectors can therefore be used to inactivate genes in discrete regions of the adult mammalian brain and offer exciting possibilities for studying gene function in the nervous system.

Our study extends the recently published studies, which have shown that AAV, LV and adeno virus expressing Cre protein can mediate loxP-dependent recombination [17,25,26] and have potential to knock out the gene of interest in the brain [27]. However, some studies have reported inflammation, growth inhibition and toxic effects of Cre in cultured cells and considerable tissue damage following Cre delivery to the brain using viral vectors [3,17,18]. In contrast, we observed the expression of Cre in cultured 293T cells without obvious toxic effects. We also found that Cre was expressed in mouse brain following injection of LV-Cre-EGFP, or a mixture of AAV-Cre and AAV-GFP vectors, into neocortex and hippocampus without any sign of cavity formation [cf [17]], in and around the injection sites. In this respect our results more

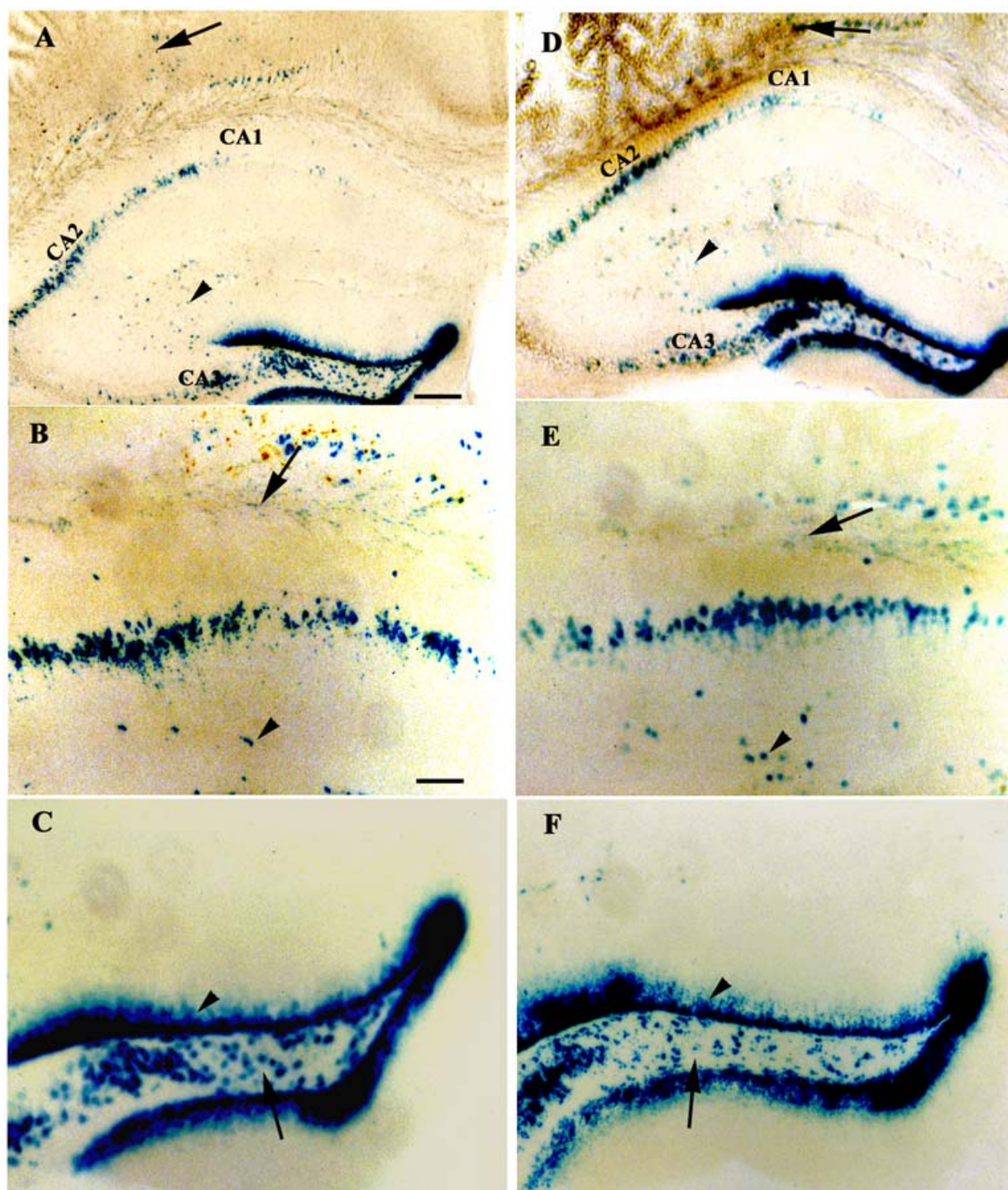


Figure 5

Cre-mediated recombination in the neocortex and hippocampus of Rosa26 reporter mice, 4 weeks following injection of LV-Cre-EGFP recombinant virus (A-C) or a mixture of AAV-Cre and AAV-GFP recombinant viruses (AAV-Cre/AAV-GFP mixture) (D-F). β -galactosidase activity is apparent in and around the needle track in the neocortex (arrows in A and D), in parts of the CA1, CA2 and terminal portion of the CA3 region of the hippocampus and in the dentate gyrus. Cells in stratum lacunosum-moleculare (arrowheads in A and D), corpus callosum (arrow in B and E) were also observed. Parts of CA1 and CA2 from Fig. 5A and 5D are enlarged in Fig. 5B and 5E and the region of the dentate gyrus from Fig. 5A and 5D is enlarged in Fig. 5C and 5F. Scale bars in A = 200 μ m (applies also to D); B = 100 μ m (applies also to C, E and F).

closely reflect those of Scammell et al. [27]. In our study the LV vector was injected in a much smaller total volume (0.5 μ l as opposed to 3 μ l) than in the Pfeifer study [17] but probably containing a similar number of viral particles (7.6×10^6 transgene expressing unit as opposed to 6×10^7 infectious unit). It has been demonstrated that toxic effects of Cre are dose dependent [18], and that low levels of Cre expression can cause significant recombination without having any adverse effects on cell viability in vitro or in vivo [25,28]. It is possible that the levels of Cre expression in the present study were sufficiently low to avoid toxic effects observed by others [[3,17,18,29] see above]. One of the advantages of Cre delivery to the CNS with viral vectors is that expression of Cre can be restricted to distinct groups of cells in the adult brain, predominantly at the site of injection, and this may also limit possible undesirable consequences of its expression. The CMV promoter was chosen because of its ability to drive expression of transgene expression in a variety of cells [30,31] but the use of neuron- or glial-specific promoters in viral vectors has the potential to further refine the use of the Cre-loxP system in studies of gene expression in the CNS.

Both neuronal and glial cells were transduced by both AAV and LV vectors, as shown by their expression of immunohistochemically detectable Cre and GFP, but neurons were the most abundant transduced cells with both viral vectors. Transduction of glial cells was most obvious in the subcortical white matter. AAV and LV vectors using the CMV promoter have been reported to produce transgene expression predominantly in neurons but to a lesser extent also in glia [9,26]. The reporter gene in Rosa26 mice is also functional in all cell types. The staining pattern obtained by immunohistochemistry after LV-Cre-EGFP and AAV-Cre/AAV-GFP mixture injections was similar, suggesting that the specificity of infection was similar. However the expression of Cre and GFP was to some extent cell type specific. For example the neurons in the dentate gyrus were more frequently transduced than most other neurons in the hippocampal formation, whereas neurons in most of the CA3 region (except the terminal part) were only very rarely transduced. This could simply reflect a difference in the ability of the viruses to infect different types of neuron in the hippocampus, perhaps because of the presence of specific receptors on the cell surface. If specific receptors for the viral vectors determine which cells are infected, our results suggest that similar receptors bind both vectors. It is possible that the small volume of the vectors injected in our experiments may have restricted the types of neurons infected.

Recombination, detected by β -galactosidase expression, was found in the same populations of cells as expressed GFP and/or Cre. Hence, recombination was predomi-

nantly in, but not restricted to, neurons and in the hippocampal formation recombination was most obvious in dentate gyrus neurons and scarcely detectable in neurons within most of CA3. β -galactosidase activity was detected in dentate gyrus neurons 3 days after injection of LV-Cre-EGFP and AAV-Cre/AAV-GFP mixture, although maximal expression of β -galactosidase was found at 14 days or more. AAV vectors carrying erythropoietin have been reported to produce expression of the transgene in striated muscle one day after infection [32] and lentiviral vectors have been shown to produce GFP expression in retinal pigment epithelium in 2–3 days [33] so that early expression of Cre would be expected using AAV-Cre. Kasper et al. [25] using an AAV vector injected into forebrain have also shown the increase of Cre activity over time. There was some evidence that AAV-Cre was more efficient at producing recombination than LV-Cre in the first 3 weeks. Even at 4 weeks after injection of either vector, fewer cells showed recombination than expressed GFP, which suggests that recombination was probably still occurring in virally transduced cells in the brains of Rosa26 mice.

Conclusion

Our data show that Cre can be effectively delivered to discrete regions of the adult mammalian brain using AAV and LV vectors. This offers the potential for producing changes in the genome of neurons in discrete regions of the brain at the time of choice. The limiting factor in exploiting this technology is likely to be the generation of strains of mice with loxP-flanked genes of interest to neuroscience.

Methods

The AAV vector pTRCGW (5.6 kb), (a kind gift from Paul Dijkhuizen, The Netherlands Institute for Brain Research, Amsterdam, The Netherlands), carrying the ampicillin resistance gene, inverted terminal repeats (ITR), human immediate early cytomegalovirus promoter (CMV), wood chuck posttranscriptional regulatory element (WPPE) and multiple restriction sites, was used to construct the rAAV-Cre vector plasmid. The 1.1 Kb fragment of Cre gene along with the nuclear localization signal (NLS) was excised from pNSE-Cre [2] by digesting with Hind III and NdeI restriction enzymes and blunted on both sites using Ecoli DNA polymerase I (klenow fragment). AAV-Cre was created by inserting NLS-Cre between two ITRs in pTRCGW vector that had been digested with SpeI and Bam HI sites, were also blunt ended by using klenow fragment.

To generate the LV-Cre-EGFP plasmid, a 2 Kb NotI/BglII fragment containing Cre-EGFP was isolated from pCMV-Cre-EGFP, blunted, and ligated into the multiple cloning site of the lentiviral transfer vector p156RRLsin-PPTh-CMV-GFP-pre [6,34,35]. This vector contains a CMV promoter along with the HIV-1-Sin 18 LTR and the HIV-1

genomic RNA packaging signal. pCMV-Cre-EGFP was created by inserting NLS-Cre derived from pJTCRE3 [35] into the multiple cloning site of pEGFP-N3 (Clontech, CA, USA).

AAV-Cre Viral vector production

Ten culture dishes (100-mm diameter), each containing 5×10^6 293T cells, were co-transfected by calcium phosphate with 5 μ g of either AAV-GFP or AAV-Cre vector plasmid and 15 μ g of the packaging plasmid (PDG) [37]. Twelve-fourteen hrs following the transfection, medium was replaced by complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% FCS (Gibco) and 1% of a mixture of penicillin (100 IU/ml) and streptomycin (100 pg/ml), in which the cells were incubated for 48 hrs at 37°C and 5% CO₂. The cells were dislodged from the culture dishes in 2 ml of PBS, pH 7.5, 1 mM MgCl₂ and 10 mg/ml DNase I and freeze-thawed three times in a dry ice/100% ethanol bath and 37°C water bath. The cell debris was spun down at 4000 rpm for 20 min at 4°C. The supernatant was passed through a heparin-agarose beads column (H-6508 Sigma, Econo column, BioRad CA, USA) followed by Iodixanol purification (Nycomed Pharma AS, Oslo, Norway) and centricon concentrator (Amicon, USA) as described previously [38]. The transgene expressing unit titre (tu/ml) was determined by co-infection of 293T cells with serial dilutions (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}) of the AAV-GFP or AAV-Cre viral vector stock in the presence of adenovirus (multiplicity of infection, MOI = 3). The titre of AAV-GFP was determined by counting GFP positive cells in a fluorescence microscope following 16 hrs infection. For detection of AAV-Cre, cells were washed with PBS, fixed with 4% PFA and processed for the immunohistochemical detection of Cre as described below. The titre was found to be 1×10^8 tu/ml for both AAV-GFP and AAV-Cre.

To determine if Cre was expressed in AAV-Cre infected cells in vitro, 293T cells were plated in 6 well plates (5×10^5 per well) in complete DMEM. Cells were infected with AAV-Cre (1:10,000) in the presence of adenovirus (MOI = 3). Fourteen-forty eight hrs following the infection, cells were washed with PBS and fixed with 4% PFA for 15 min, followed by washing with PBS (3×10 min). Cells were then incubated with antibody to Cre (1:200, Berkeley, CA, USA), diluted in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and 0.1% Triton) overnight at 4°C. The following morning, cells were washed with PBS (3×5 min) and incubated with ABC solution (Avidin Biotin Complex, Vector laboratories, CA USA) for an hour at room temperature. The cells were washed with PBS (3×5 min) and allowed to react for 5 min in a solution of 0.05% diaminobenzidine and 0.03% hydrogen peroxide in PBS. The cells were washed with PBS to terminate the reaction and subse-

quently observed under a bright-field microscope (Zeiss Axiovert 10).

LV-Cre-EGFP viral vector production

For viral vector production, the LV-Cre-EGFP transfer plasmid was co-transfected together with the viral core packaging construct pCMVdeltaR8.74 and the VSV-G envelope protein vector pMD.G.2 into 293T cells as previously described [39]. The original transfer vector and the helper plasmids were obtained from Dr. L. Naldini (Institute for Cancer Research, University of Torino, Italy). Briefly, 5×10^6 293T cells were seeded in 10 cm dishes 24 hrs prior to transfection in complete Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, glutamine and 1% penicillin/streptomycin in a 5% CO₂ incubator, the culture medium was changed 2 hrs prior to transfection. Ten μ g of transfer vector plasmid, 6.5 μ g envelope plasmid, and 3.5 μ g of packaging plasmid were co-transfected using the calcium phosphate method. The medium was replaced after 14–16 hrs and the conditioned medium was collected 24 hrs later, cleared by low-speed centrifugation (1000 rpm for 5 minutes) and filtered through 0.22 μ m cellulose acetate filters. The supernatant was concentrated about 100 fold by ultra centrifugation (20,000 rpm for 2.5 hrs). The pellet was re-suspended in PBS and virus was aliquoted and stored at -80°C. The titre was determined by infecting 293T cells with serial dilution of the LV-Cre-EGFP virus (10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}) in complete IMDM. Cells were incubated for 16 hrs and the medium was replaced with fresh IMDM and cells were incubated for 24 hrs. The titre was determined by counting GFP positive cells under a fluorescence microscope. This was confirmed by counting positive cells after Cre-immunocytochemistry as described above. The titre of the first stock was 1.5×10^{10} tu/ml and the second stock was found to be 2×10^9 .

To determine if Cre was expressed in LV-Cre-EGFP infected cells in vitro, 293T cells (5×10^5 per well) were plated in complete IMDM. The next day, cells were infected with LV-Cre-EGFP (1:10,000) in complete IMDM medium for 16 hrs, medium was replaced with fresh medium and incubated for 24–48 hrs. These experiments were replicated 3 times. For immunofluorescence labeling, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min, followed by washing with PBS (3×10 min). Cells were then incubated with the Cre antibody (1:200, diluted in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and 0.1% Triton) overnight at 4°C. Next morning cells were washed with PBS (3×10 min) and incubated with Cy3-conjugated goat anti-mouse (1:400, Jackson laboratories PA, USA) for an hour at room temperature. Cells were washed with PBS (3×10 min), mounted on slides and observed under a confocal microscope (LSM

410 Zeiss) using 488 nm and 543 nm excitations for GFP and Cy3 respectively.

AAV and LV delivery of Cre into the brain

Rosa26 reporter mice (22–30 g) were deeply anaesthetised by a fluanisone /fentanyl mixture containing Hypnorm (Janssen Pharmaceutical, UK) and Dormicum (Roche, NL) and water in a ratio of 1:1:2 (10 µl/gm I.P.). Virus was injected into the cortex and hippocampus via 30G stainless needle attached by polyethylene tubing to a 10-µl Hamilton syringe and a Harvard 22 microinjection pump. 0.8 µl of a mixture of AAV-Cre and AAV-GFP (AAV-Cre/AAV-GFP mixture, 9:1) or 0.5 µl LV-Cre-EGFP virus was injected into the cortex (anterioposterior -2, lateral + 1.3, dorsoventral -0.45) and hippocampus (anterioposterior -2, lateral + 1.3, dorsoventral -1.55) [40] at a rate of 0.2 µl/min. The needle remained in place for an additional 5 minutes to facilitate the controlled delivery of virus. Three days and 1, 2 and 4 weeks following the injections mice were perfused with 4% PFA. Four mice were examined for each time point. Sections of the brain were cut in the coronal plane at a thickness of 50 µm with a vibratome and processed for β-galactosidase enzyme histochemical, immunohistochemical, and double immunofluorescence studies.

β-galactosidase staining was done by incubating the sections for an hour at 37°C with X-gal-solution containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside in PBS. The X-gal reacted sections were washed with PBS (3 × 10 min), dehydrated and embedded in entellan mounting medium. Immunohistochemistry for GFP was carried out by the ABC method using diaminobenzidine as a substrate [41], and with rabbit anti GFP (1:100, Chemicon, CA, USA) as primary antibody and biotinylated anti-rabbit (1:200, Vector) as secondary antibody.

Colocalization of Cre and GFP was examined by using a double immunofluorescence. A mixture of biotin labelled monoclonal Cre antibody (1:1000) and polyclonal GFP antibody (1:100) was used as primary antibodies, while a mixture of streptavidin-conjugated horseradish peroxidase (1:200, Life Sciences, MA, USA) and FITC conjugated anti-rabbit (1:100, Sigma) was used as secondary antibodies. Cre signal was enhanced by incubating the sections in fluorophore labelled tyramide solution (1:200, Life Sciences) for 30 min at RT, mounted sections were analyzed by fluorescence microscope fitted with SP confocal head (Leica DMRE) using Leica confocal software.

Author's contributions

BYA generated the Cre vectors in collaboration with WTJMCH and SN under the supervision of JV. The Cre-EGFP fusion construct was cloned and the Cre reporter

mice provided by CL. The in vitro and in vivo studies were carried out by BA in collaboration with RE and SC. The histological work was carried out by BYA and PNA. ARL, FS, JZ and PNA participated in the design of the study and performed part of the histological analysis. FS, JV, PNA and ARL conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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